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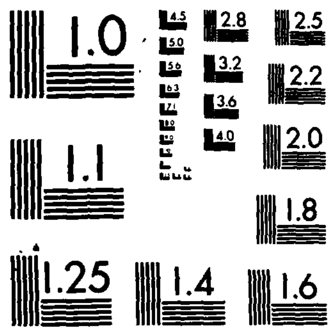

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LIPOPROTEIN AGAROSE GEL ELECTROPHORESIS: APPLICATION IN HDL-CHOLESTEROL METHODOLOGY

Emmanuel L. Mosser, B.S.
Dale A. Clark, Ph.D.

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Aerospace Medical Division (AFSC)
Brooks Air Force Base, TX 78235-5301

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NOTICES

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nations.

This report has been reviewed and is approved for publication.

Emmanuel L. Mosser

EMMANUEL L. MOSSER, B.S.
Project Scientist

Julio E. Acevedo

JULIO E. ACEVEDO, Colonel, USAF, MC
Supervisor

Royce Moser, Jr.

ROYCE MOSER, Jr.
Colonel, USAF, MC
Commander

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)
We perform agarose gel lipoprotein electrophoresis (AGLE) in this laboratory using glass microscope slides to support the agarose gel and 0.025 M barbital buffer for the electrophoresis. This report describes details, including special apparatus used to facilitate making and handling agarose gel slides.

To test correlations between serum lipid levels and the densities of the corresponding lipoprotein bands separated by electrophoresis, we electrophoresed eight individual sera and measured the densities of the resulting lipoprotein bands. Serum levels of total cholesterol, triglycerides, and HDL-cholesterol were correlated with the measured densities of the beta, prebeta, and alpha lipoprotein bands respectively. Coefficients for correlation between total cholesterol and beta lipoprotein, triglyceride and prebeta lipoprotein, and HDL-cholesterol and alpha lipoprotein were 0.638, 0.914, and 0.920 respectively. Densitometric data from 24 replicate electrophoresis slides showed that the coefficient of variance for the
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19. Abstract (continued)

percentage of dye bound to the beta, prebeta, and alpha lipoproteins was 1.9%, 2.6%, and 4.5% respectively.

We used AGLE to monitor the separation of high-density lipoprotein (HDL) from the combined low-density lipoproteins (LDL) and very low density lipoproteins (VLDL) during the titration of serum lipoproteins with increasing amounts of lipoprotein precipitant. Results demonstrated which levels of precipitant removed all detectable VLDL + LDL and which levels of precipitant removed a significant amount of HDL. These data, together with the levels of cholesterol in the supernate from the various tubes, identified the optimum precipitant volumes to be used in routine determinations of serum HDL-cholesterol levels.

LIPOPROTEIN AGAROSE GEL ELECTROPHORESIS: APPLICATION IN HDL-CHOLESTEROL METHODOLOGY

INTRODUCTION

In recent years the interest in high-density-lipoprotein cholesterol (HDL-C) as a strong inverse risk factor for coronary artery disease (1,2) prompted the development of methodology for measuring the level of HDL-C in serum. This level is commonly measured in the supernate obtained after adding a precipitating reagent (3,4,5) to an aliquot of serum to remove all lipoproteins except the HDL. Quantitative measurement of cholesterol in the supernate, however, does not establish that the cholesterol was provided only by HDL. Lipoprotein electrophoresis can be used for this purpose. In addition, when used in conjunction with determinations of cholesterol levels in supernates obtained after addition of various amounts of precipitants, lipoprotein electrophoresis can help to establish the optimal amounts of precipitating reagent needed for accurate determination of serum HDL-C levels. This technique has been used in this laboratory for several years. Our procedures for lipoprotein electrophoresis include several adaptations of published methods, so this report will describe the method in detail and its application in determining the optimum volume of precipitating reagent for use in measuring levels of serum HDL-C.

MATERIALS AND METHODS

Materials: The sources of materials are listed in Table 1.

Specimens: Blood was drawn from human subjects after an overnight fast. After a clot formed, the blood was centrifuged and the serum was collected and stored at 4°C. Electrophoresis was performed within the next 48 h.

Lipoprotein electrophoresis. The lipoprotein agarose gel electrophoresis method described herein is a modification of methods reported by others (6,7,8). The major modification is that we used glass microscope slides for agarose gel support; we also made small changes in the preparation of the Oil Red O stain and in densitometry.

Preparation of the Agarose-Ionagar-Albumin solution. The concentrations of agarose-ionagar-albumin are those of Noble (6), except that the barbital buffer (7) used to dilute the agarose and the ionagar was 0.025 M, pH 8.6. The following directions apply. Albumin solution: Using a 30% human albumin solution obtained from a commercial source, prepare a 25% albumin solution (6) by adjusting the pH of 41.5 ml with 1 M Tris buffer to pH 8.6, and then diluting to 50 ml with 0.9% NaCl solution. Agarose and Ionagar solutions: Weigh 200 mg agarose and 90 mg ionagar into separate 125-ml Erlenmeyer flasks and add 40 ml and 15 ml barbital buffer respectively. Stopper the flasks with a roll of 10 X 20 cm (4" X 8") gauze and place in a boiling water bath with occasional twirling fcr 5 min. Bring the solutions to a boil while twirling

TABLE 1. SOURCES OF MATERIALS USED

Agarose Powder Cat.No. 162-0100 1% Bromphenol Blue	Bio-Rad Laboratories 32nd and Griffin Ave. Richmond, CA
Ionagar No. 2 Code No. L12	Consolidated Laboratories, Inc. Box 234 Chicago Heights, IL
30% Human Albumin Code No. 82-305-1	Miles Laboratories P.O. Box 2000 Elkhart, IN
Barbital Buffer Item No. 3948	Harleco Gibbstown, NJ
Oil Red O Dye, Powder	Hartmen-Leddon Co. Philadelphia, PA
ABA 100	Abbott Laboratories 820 Mission St. South Pasadena, CA
Remote Control Pipette Micro Model, Cat. No. 34 182011	Lapine Scientific Co. Chicago, IL
20-μl Disposable Pipettes Cat. No. 21-164-2D	Fisher Scientific Co. Pittsburgh, PA
Densitometer Clinical Scanning Densitometer System Models 2500/2510	Transidyne General Corp. Ann Arbor, MI
Wicks, Filter Paper Spinco Part No. 300-29 Spinco Model R Durrum-type Cell Spinco Model RD-2 Duostat Power Supply	Beckman Instruments, Inc. Palo Alto, CA

NOTE: The citation of these items and their suppliers does not constitute product endorsement by the U.S. Air Force nor does it imply superiority over comparable items from other sources.

over an open flame. Place the clear solutions (no granules of agarose or ionagar) in a water bath at 50°C and allow to equilibrate to that temperature. Agarose-ionagar-albumin solution: Mix together the prepared agarose, ionagar, and albumin solutions in the volumetric proportions of 40:10:1.

Well-Formers. A glass microscope slide holder and well-former combination was designed (Fig. 1). Each slide fits in a depression machined into the Plexiglas base. The depression is slightly wider than the width and 3.2 mm (1/8") shorter than the length of a microscope slide. The depth of the depression is approximately one half the thickness of the slide. There are eight such depressions in the base. At each end of the base there is a support block appropriately grooved to hold one end of a square support rod. To that rod, eight metal well-formers (6), each 2 mm thick X 20 mm wide X 37 mm long, are attached by eight screws, each of which passes through a slot in the upper end of a well-former. These screws are used to adjust the height of each well-former so that its lower end contacts a glass slide placed below it. The lower end of the well-former, which contacts the glass slide, is oval shaped; its diameter is 2 mm at the thickest point. A clean glass microscope slide, marked for identification on the back side with a diamond-point pencil, is placed in each depression; and each well-former is adjusted so that the lower end rests on its microscope slide. After the apparatus is adjusted to level the base, the square rod with attached well-formers is removed for the application of agarose to the glass slides.

Preparation of Agarose Gel Slides. Working with one slide at a time, carry out the following procedure on each slide mounted on the base. Using a warm 5-ml pipette graduated to the tip, deliver 1.5 ml of warm (50°C± 2°) agarose solution onto the glass slide with a zigzag motion. Use the pipette to quickly spread the agarose around the outer edges of the slide to ensure uniform coverage of the entire slide. When each slide has been covered with agarose solution, replace the square rod with attached well-formers and let the unit sit, first at room temperature for 15 min, then in a dry heat incubator at 37°C for 15 min, followed by a return to room temperature. Remove the square rod with its well-formers, leaving the agarose-coated slides on the base. The sample wells produced with this combination of apparatus and procedures are located 18 mm from the end of the glass slide and have an optimum capacity of 20 µl.

Dilution of Sample. Deliver 200 µl of sample near the bottom of a 12- X 75-mm test tube and place in a 50°C water bath. Add 100 µl agarose-ionagar-albumin solution to the sample in the tube, and vortex for a few seconds. Cap the tube and return it to the rack in the 50°C water bath until used for electrophoresis.

Application of Sample and Tracking Dye. Holding a remote-control pipette unit in the left hand and an attached 20-µl disposable pipette in the right hand, remove excess fluid from each slide well. Use the same apparatus to deliver 20 µl of sample mixture into each slide well. Prepare two tracking-dye slides without sample wells for each electrophoresis cell. To each of these slides, apply a 1% aqueous solution of bromphenol blue. Using the sharp end of a broken wood applicator stick, apply this tracking-dye solution as a line perpendicular to the length of the slide, approximately 18 mm from one end of the slide. Use a wax pencil to mark a line on the back side of the slide 35 mm from the point of dye application.

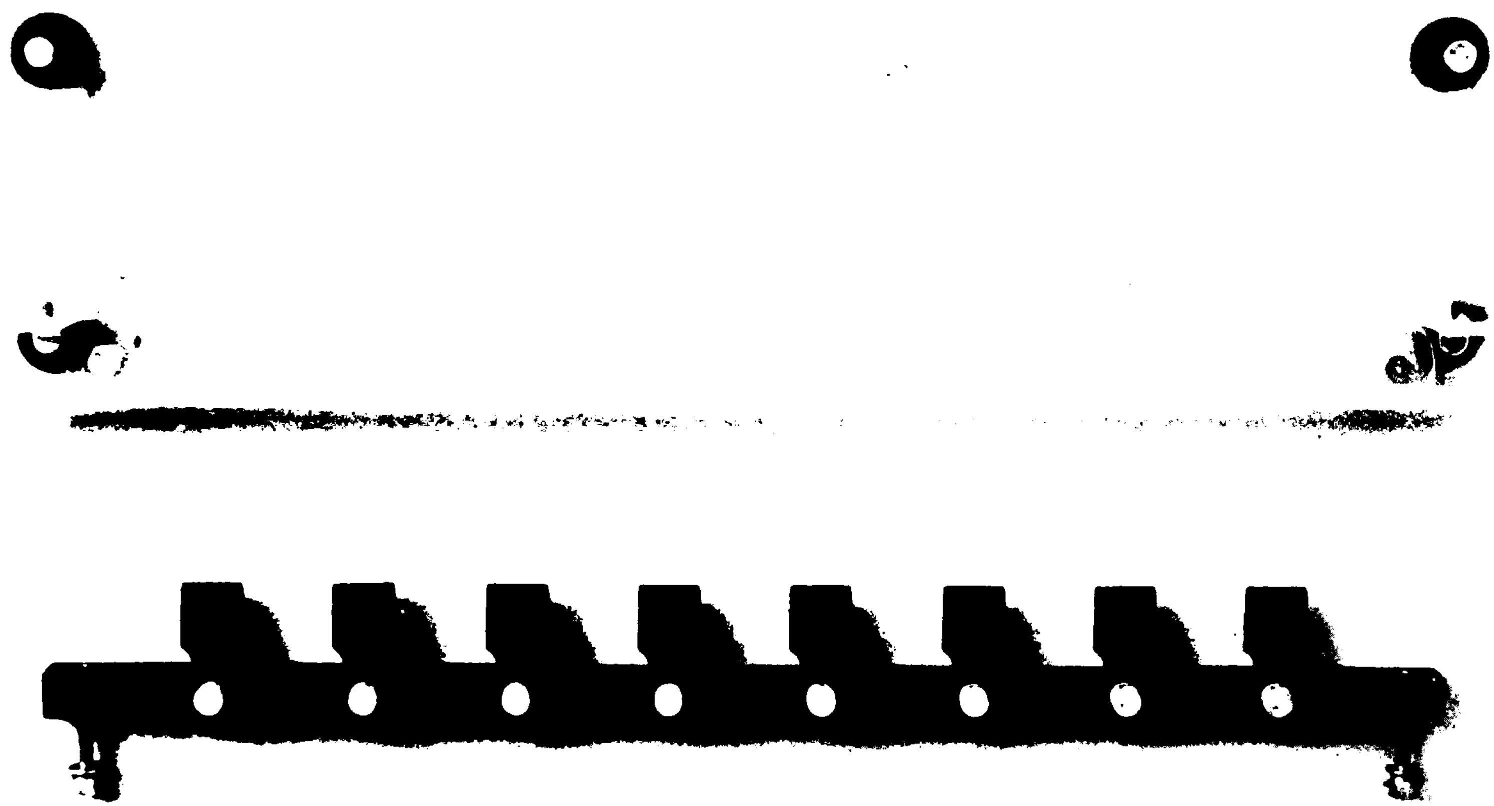

Figure 1. Slide holder and well-former combination.

Electrophoresis. A Spinco Model R Durrum-type cell (7) and a Beckman RD-2 Duostat power supply were used for the electrophoretic separation. The Durrum cell racks for holding paper strips were removed, but the partitions and wick holders were left in place. Two wicks were used on each side of the Durrum cell, but their width was reduced 6.4 mm (1/4") by cutting off a 6.4-mm strip from one side of each wick. One wick was positioned in the wick holder and saturated with buffer. A second wick was inserted alongside the first so that approximately 9.5 mm (3/8") extended above the wick holder. Two wicks were positioned similarly on the other side of the Durrum cell. After the wicks were saturated with buffer, the 9.5-mm extension above the holder was folded over in a 90° angle toward the center of the cell. The wick holder was stabilized by thin O-rings of plastic tubing wedged between ends of the wick holder and the cell. A sheet of plastic 3.2 mm X 76.2 mm X 30.5 cm (1/8" thick X 3" wide X 12" long) was laid atop the folded-over wicks and weighted down with a plastic block 2.54 X 4.74 X 30.5 cm (1" thick X 1-3/4" wide X 12" long). These pieces of plastic were left in place for 24 h for saturation/equilibration of the wicks before electrophoresis. The weighted plastic prevented buckling of the folded-over wicks so that they would provide a flat area for contact with the agarose gel slides subsequently placed on them. Immediately before electrophoresis, the plastic sheet and bar were removed. The agarose gel slides were placed, gel side down against the wick, with the slides spanning the distance between the anode and cathode compartment wicks and the sample or dye application point closest to the cathode. A tracking-dye slide was placed at each end of the cell. The plastic bar was laid atop the slides, midway between the wicks and parallel with them. Electrophoresis was carried out at room temperature at 5-mA current per slide until the dye on the tracking-dye slides had migrated past the wax-pencil marking 35 mm from the starting line. The time required was usually 35 min. Following electrophoresis, the slides were placed in a fixing solution (9) of ethanol, glacial acetic acid, and distilled water (60:5:35::v:v:v) for 15 min, then in another batch of the same fixing solution for another 15 min. The slides were then dried in a dry-heat oven at 80-85°C, usually for 45 to 60 min.

Staining and Densitometry. Oil Red O stain was prepared by the method of Jencks (10) except that the dye mixture was stirred overnight at 45°C, filtered at 34°C, and stored at 37°C. After being fixed as described above, the slides were arranged in stainless-steel racks and stained in the Oil Red O solution at 37°C for 24 h. They were then washed thoroughly in a stream of distilled water and dried at 37°C. Densitometric scanning of the dried stained slides was performed at 520 nm, using a Transidyne General Corporation densitometer.

Chemical Analyses. Total cholesterol levels were measured using an ABA-100 Bichromatic Analyzer and a commercial enzymatic reagent. HDL-C levels were measured using the same method applied to the supernate obtained after precipitation of LDL and VLDL by our phosphotungstate-$MgCl_2$ (11) technique. Triglyceride levels were measured with the same ABA-100 using a commercial enzymatic triglyceride reagent.

RESULTS

To explore variations among individual serum samples, eight serum samples chosen at random were electrophoresed as previously described. Figure 2 shows the results obtained. The electrophoresis patterns demonstrate the separations of the alpha, prebeta, and beta lipoprotein bands separated by the methods described in this paper. The strip charts are the corresponding densitometric readouts. Because the uptake of Oil Red O dye by the lipoprotein fractions is a function of the lipids in the lipoproteins and because the serums used have various levels of cholesterol, HDL-C, and triglyceride, the densitometric data and the serum lipid levels are listed in Table 2. As expected, the densitometric data tend to vary with the relevant lipid levels. Correlations found between lipid levels and electrophoresis band densities are listed in Table 3. To evaluate the reproducibility of the agarose gel lipoprotein electrophoresis procedure, 24 aliquots of a single serum were similarly electrophoresed. The reproducibility found is summarized in Table 4. The coefficients of variation found were relatively small, in part because of the good separation between bands (cf. Fig. 2).

TABLE 2. SERUM LIPID LEVELS AND LIPOPROTEIN ELECTROPHORESIS DATA

	Lipids (mg/dl)			Densitometric electrophoresis units			% dye bound		
Serum No.	Total Chol	Trig	HDL Chol	Beta	Prebeta	Alpha	Beta	Prebeta	Alpha
1	152	42	69	11.03	2.01	10.86	46.1	8.4	45.4
2	204	64	76	12.91	3.38	10.51	48.1	12.6	39.2
3	181	121	45	15.05	4.92	7.42	54.9	18.0	27.0
4	344	294	36	16.51	9.56	3.84	55.2	31.9	12.8
5	249	124	37	14.17	2.82	4.23	66.7	13.3	19.9
6	173	82	45	15.29	2.15	6.93	62.7	8.8	28.4
7	260	155	45	14.58	5.06	4.53	60.3	20.9	18.7
8	167	112	46	13.67	5.65	7.26	51.4	21.2	27.3

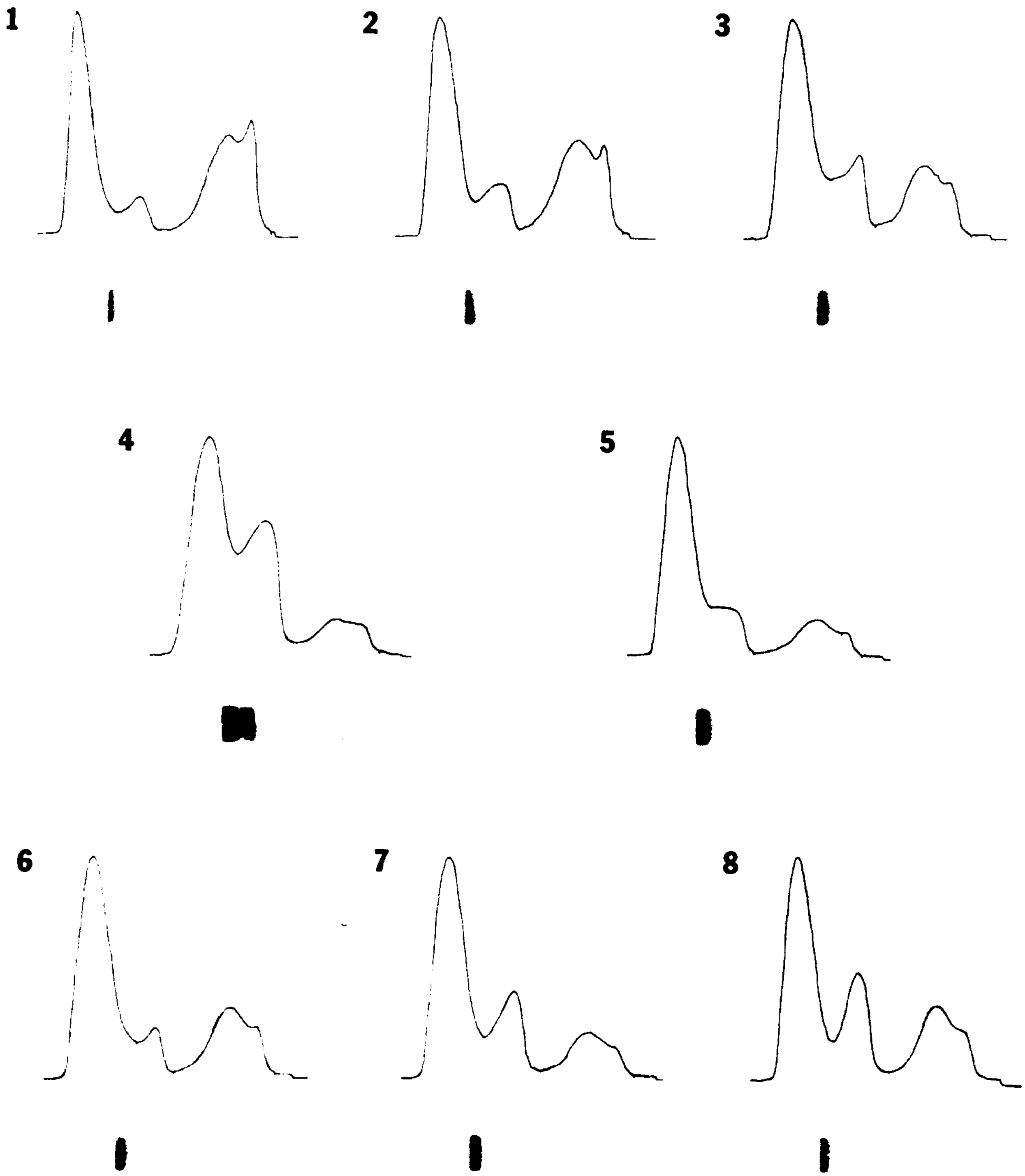

Figure 2. Electrophoresis of random serum samples. The densitometric tracing is shown above its corresponding electrophoresis slide for each of the eight serum samples.

TABLE 3. CORRELATIONS BETWEEN SERUM LIPID LEVELS AND RESULTS OF QUANTITATIVE LIPOPROTEIN ELECTROPHORESIS

	Observed Coefficients			Published Coefficients		
Lipoprotein	Total chol	Trig	HDL chol	Total chol	Trig	HDL chol
Beta	0.638	--	--	0.673[a]	--	--
Prebeta	--	0.914	--	--	0.972[a]	--
Alpha	--	--	0.920	--	--	0.947[b]
N	8	8	8	16	20	98

[a]Hulley, reference 8.

[b]Conlon, reference 13.

TABLE 4. QUANTITATIVE LIPOPROTEIN ELECTROPHORESIS: PERCENTAGE DYE BOUND TO LIPOPROTEIN FRACTIONS

	Beta	PreBeta	Alpha
Mean %	59.5	20.6	19.8
Range %	57.4 - 61.5	19.3 - 21.3	18.4 - 21.3
N	24	24	24
1 S.D.	1.13	0.54	0.89
CV	.0190	.0262	.0449

In lipoprotein titration experiments in this laboratory, agarose gel lipoprotein electrophoresis is routinely performed on the supernatant solutions obtained from the titration tubes (12). The results from one such experiment will illustrate the information obtained from such titrations. In this particular experiment, we added 1.0-ml aliquots of a single serum sample to each of a series of test tubes. To each tube we then added 200 μl of precipitant solution, but the composition of that solution varied systematically from tube to tube. The proportion of the 200 μl made up of phosphotungstate-$MgCl_2$ precipitating reagent increased from 0 to 100% while the proportion made up by normal saline correspondingly decreased from 100% to 0 in progressing from the first to the last tube in the series (cf. Table 5). After the serum-precipitant solution was mixed, we centrifuged the mixture at 1400 x g for 30 min at 4°C. We decanted the supernatant solution from each tube in the series into a clean test tube and took aliquots for agarose gel lipoprotein electrophoresis and for cholesterol determination. Results of this titration experiment are presented in Figure 3, which is a plot of the cholesterol content of the supernatant against the varying volumes of precipitating reagent added. Densitometric data from the agarose gel electrophoresis slides together with the cholesterol content of the corresponding tube are given in Table 5. A plateau occurred in the cholesterol level in tubes 6-9 and another in tubes 10-12. The electrophoresis data show that all the beta and prebeta lipoproteins had been precipitated in tubes 6-13 and suggest some possible loss of HDL from the alpha band in tubes 10-12.

TABLE 5. HDL SUPERNATANT DATA

Tube No.	Precipitant volume (μl)[a]	Chol (mg/dl)	Densitometric electrophoresis units: Alpha	Beta + Prebeta	Total	Percentage: Alpha	Beta + Prebeta
1	0	284	566	1836	2402	24	76
2	24/6	278	521	1712	2233	23	77
3	40/10	170	510	1217	1727	30	70
4	56/14	60	800	139	939	85	15
5	64/16	52	691	74	765	90	10
6	72/18	50	691	22	713	97	3
7	80/20	50	796	14	810	98	2
8	88/22	50	570	31	601	95	5
9	96/24	50	569	46	615	92	8
10	104/26	48	537	36	573	94	6
11	120/30	47	544	31	575	95	5
12	136/34	47	516	58	574	90	10
13	160/40	41	450	42	492	92	8

[a]Volume of phosphotungstate/volume of 2M $MgCl_2$. Total volume added (phosphotungstate plus $MgCl_2$ plus saline) equals 200 μl.

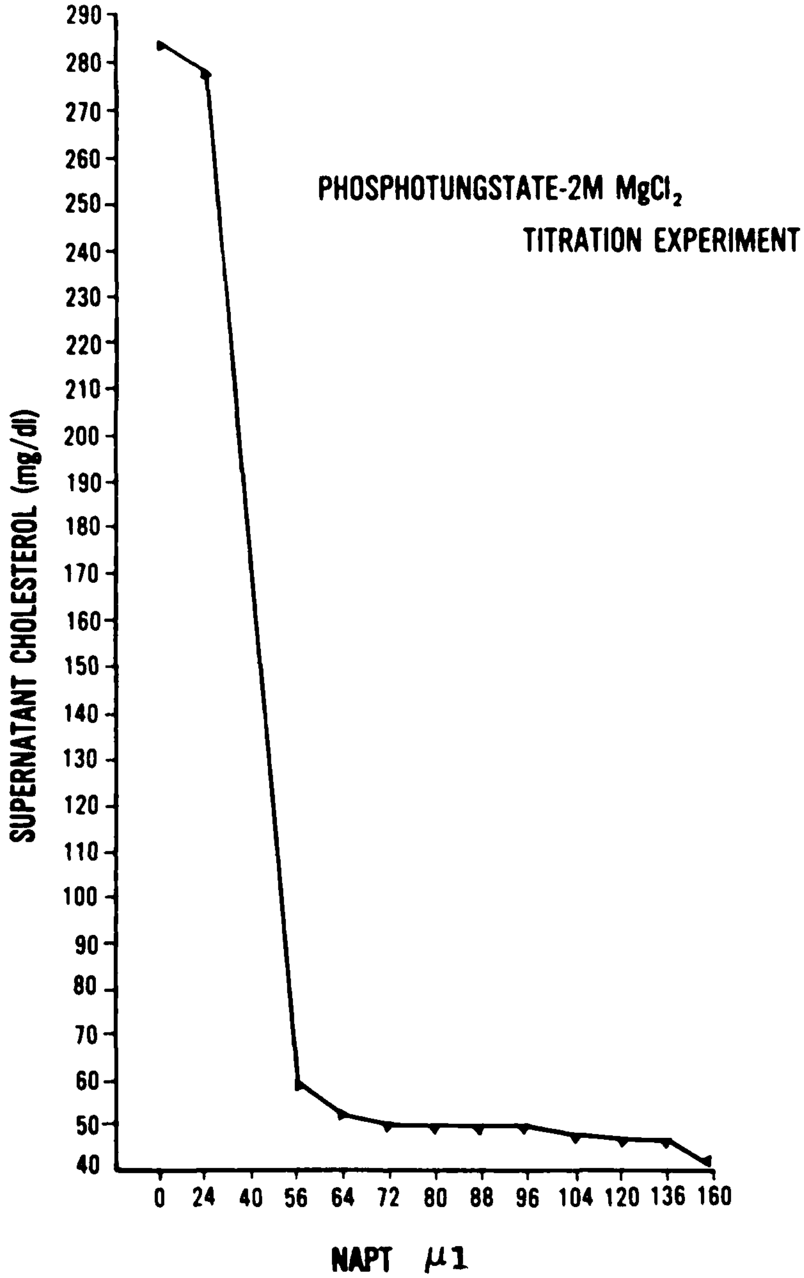

Figure 3. Cholesterol levels in the supernates are plotted against the corresponding volumes of sodium phosphotungstate-$MgCl_2$ (NAPT) precipitant added.

The closest approach to the ideal situation of precipitating all the beta (LDL) and prebeta (VLDL) lipoprotein fractions without losing any of the alpha (HDL) fraction appears to be in tubes 6 and 7, with the corresponding level of cholesterol in the supernate being 50 mg/dl. This concentration of cholesterol, therefore, is the best estimate of the true HDL-C level.

DISCUSSION

One aspect of assessing the usefulness of agarose gel electrophoresis of serum lipoproteins is an evaluation of the qualitative relationships between visual observations of the slides (Fig. 2) and the corresponding densitometric readouts (Table 2). Good agreement between those parameters is apparent. For instance, in pattern 1 (Fig. 2) the beta and prebeta bands appear to be less than average in intensity, and the same is true for the alpha band in pattern 4. For those patterns, the values for both the densitometric electrophoretic units (DEUs) and the percentage of dye bound agreed well with the visual observations.

Such agreements are confirmed by the correlation coefficients in Table 3. Those coefficients are similar to the ones reported by Hulley (8) for beta and prebeta lipoproteins and by Conlon (13) for alpha lipoproteins. For comparing triglyceride levels with prebeta DEU scores, and HDL-C levels with alpha DEU scores, the correlation coefficients (.914 and .920 respectively) are highly significant ($P<.005$). For comparing total cholesterol with beta DEU scores, the correlation coefficient of .638 is only marginally significant ($.05<P<.1$). This fact is probably related to the finding that the total cholesterol levels are inversely correlated with the alpha DEU scores ($r = -.752$, $.025<P<.05$). When LDL-cholesterol levels (estimated as total cholesterol minus HDL-C) are compared with beta DEU scores, the correlation coefficient is somewhat larger ($r = .722$) and is statistically significant ($P<.05$). The inverse relationship between total cholesterol level and alpha DEU score may be the reason that the sum of the cholesterol and triglyceride levels does not correlate significantly with the sum of the DEU scores for all three lipoprotein bands ($r = .453$, $P>.1$). However, the ratio total cholesterol/HDL-C is highly correlated with the ratio beta DEU score/alpha DEU score ($r = .980$, $P<.001$). These correlations indicate satisfactory agreement between the quantitative serum lipid levels and the results of agarose gel lipoprotein electrophoresis.

Table 4 shows the reproducibility obtained on 24 replicate electrophoresis slides prepared from sample 7 (Fig. 2 and Table 2). Both the percentage of dye bound in each band and the associated coefficient of variation (CV) of replicate runs would probably differ with other serum samples, depending on whether the bands were well separated, or whether the edges of the bands were distinct, and whether the density of each band was sufficient for accurate densitometric measurement. The CVs of 0.019, 0.026, and 0.045 for the beta, prebeta, and alpha bands, respectively, are well within the acceptable range.

In routine clinical laboratory operations, the HDL-C level is the cholesterol level measured in the supernatant solution obtained after the addition of a specified amount of lipoprotein precipitant to a specified volume of serum. If that specified amount of precipitant is optimal, only

HDL will remain in the supernate, all LDL and VLDL having been precipitated. To identify the optimal volume of precipitant, we titrated serum lipoproteins with lipoprotein precipitant and used agarose gel lipoprotein electrophoresis to monitor the supernatant solutions to determine when the LDL and VLDL had been precipitated or when too much precipitant had been added, causing some HDL to precipitate. Results of one such experiment are graphed in Fig. 3 for comparison with the corresponding quantitative data in Table 5. Ideally, as larger quantities of precipitant were added, the cholesterol level in the supernate should have decreased until all VLDL and LDL had been precipitated, then plateaued at some level until enough precipitant had been added to precipitate some of the HDL and cause the cholesterol level in the supernate to decrease further. During this sequence, the DEU scores for the beta-plus-prebeta fractions ideally should have decreased from a high initial level and reached zero when the plateau in the cholesterol level was first reached. Ideally also, the DEU scores for the alpha fraction should have been constant for all tubes in the titration series until sufficient precipitant had been added to precipitate some of the HDL.

In the laboratory, however, these ideals may be approached but never realized. The data in Table 5 illustrate the practical realities. The beta-plus-prebeta DEU scores decreased successively in progressing from tubes 1 through 5 and appeared to be roughly constant thereafter. Presumably some lipid/lipoprotein fragment left behind by the alpha band migrating ahead of the prebeta and beta bands accounted for the variability of the beta-plus-prebeta DEU scores for tubes 6-9. It seems likely that the larger amounts of precipitant in tubes 9-13 rendered some HDL marginally precipitable and increased the amount of lipoprotein fragments left behind as the HDL band migrated, causing the DEU scores of the slower beta and prebeta bands to be higher. The average DEU score for the alpha band was near 560, but the values for tubes 4-7 were higher. Possible causes for these higher scores include insufficient sample dilution, incomplete mixing of sample with agarose, or delivery of more than 20 µl of sample mixture into the sample well. Such variations would increase the DEU scores but would not affect the percentage of dye bound by the various bands. The percentage of dye bound by the alpha fraction increased in progressing from tube 1 to tube 5, remained roughly constant from tube 6 to tube 11, then decreased in tubes 12 and 13. The failure of the alpha band to account for 100% of dye bound in tubes 6-13 is probably caused by the trailing of lipid/lipoprotein fragments from the alpha band referred to previously.

Taken together, the electrophoretic data indicated 1) that the LDL and VLDL precipitation was complete in tubes 6-13, and 2) that the HDL did not begin to precipitate until tube 10. The cholesterol concentration in the supernates of tubes 6-9 should therefore be the best measure of the HDL-C level. Since the cholesterol concentrations in those tubes were all 50 mg/dl, that level was the HDL-C value for that serum. All these data supported the conclusion that the precipitant volumes used in tube 7 (which was at the middle of the plateaus in cholesterol level and maximum percentage of dye bound in the alpha fraction) were optimal for this serum.

Since tube 7 was at the center of the plateaus in the supernate cholesterol concentration and in maximum percentage of dye bound in the alpha band, the associated volumes of precipitant (80 µl phosphotungstate plus 20 µl $MgCl_2$ per ml of serum) was the dilution of choice for this serum.

These results illustrate the essential role of agarose gel electrophoresis in establishing the optimum volume of precipitant to be used in routine determinations of serum HDL-cholesterol levels.

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